



UPPSALA  
UNIVERSITET

# Production and characterization of Amyloid beta-binding Affibody molecules

Linnéa Pettersson

---

Degree project in biology, Master of science (2 years), 2012

Examensarbete i biologi 45 hp till masterexamen, 2012

Biology Education Centre, Uppsala University, and Molecular Biotechnology at Royal Institute of Technology

Supervisors: Hanna Lindberg and Dr. John Löfblom

External opponent: Ken Andersson

## **Production and characterization of Amyloid-beta binding Affibody molecules**

### **Abstract**

Amyloid beta (A $\beta$ ) peptides are believed to be one of the contributing factors to Alzheimer's disease. They tend to aggregate and form plaques, which are known to be toxic to cells. The quest of finding new treatment plans for Alzheimer's patients is ongoing and many potential drugs have failed in human trials. Humanized antibodies are one of the big players in this field, but instead of using antibodies other affinity proteins can be used, such as the Amyloid beta binding Affibody molecule. This molecule derives originally from the Staphylococcal surface protein A, where one of the domains has affinity to Immunoglobulin G (IgG). This domain has been isolated and maturation libraries can be made so the affinity is altered towards other proteins, such as the A $\beta$ -peptide. The Affibody molecule is much smaller than an antibody and might be able to penetrate the blood-brain barrier, which is an advantage when you target proteins in the central nervous system.

The original A $\beta$  binding Affibody molecule, Z<sub>A $\beta$ 3</sub>, has previously been selected as monomer, which is the standard way to select for Affibody molecules. NMR showed however that it binds the A $\beta$ -peptide as a dimer with a hydrophobic cleft. The A $\beta$  binding Affibody molecule was later engineered as head-to-tail dimer. This binder contains cysteine and removal of these results in reducing the affinity, suggesting that the cysteine is important for binding of the A $\beta$ -peptide. Two maturation libraries of the head-to-tail engineered A $\beta$  binding Affibody molecule were done in order to increase the affinity towards the A $\beta$ -peptide and the top eight candidates were chosen to do further studies on and characterize.

The aim of this project was to produce and characterize eight Amyloid beta binding Affibody molecules, with two A $\beta$ -binding Affibody molecules as controls, from an affinity maturation library aimed for potential therapeutic and diagnostic applications.

All A $\beta$  binding Affibody molecules were successfully produced and characterized both in free form and on *Staphylococcus carnosus* surface. The affinity has been increased a 100 fold when comparing to the original A $\beta$  binding Affibody molecule, Z<sub>A $\beta$ 3</sub>. Also, preliminary results suggest that the A $\beta$  binding Affibody molecule inhibits the plaque formation *in vitro*.

Further studies have to be done in order to characterize these binders even more. Some of the top candidates from this project will be tested in mouse models both for imaging and as a therapeutic drug.

## Index

<b>INTRODUCTION</b> .....	<b>4</b>
ALZHEIMER'S DISEASE .....	4
CURRENT AND FUTURE TREATMENT STRATEGIES .....	5
AFFIBODY MOLECULE .....	6
AMYLOID BETA TARGETED AFFIBODY MOLECULE .....	6
STAPHYLOCOCCAL CELL SURFACE DISPLAY .....	7
BACKGROUND .....	8
AIM .....	8
WORKFLOW .....	8
<b>MATERIALS</b> .....	<b>9</b>
BUFFERS .....	9
BACTERIAL STRAINS .....	10
EQUIPMENT .....	10
SOFTWARE .....	10
<b>METHODS</b> .....	<b>11</b>
STAPHYLOCOCCUS DISPLAY .....	11
ISOLATION AND PURIFICATION OF ZABETAMATLIB CONSTRUCTS .....	11
INSERTION AND EXPRESSION OF VECTORS .....	12
PURIFICATION WITH IMAC AND SEC .....	13
VERIFICATION OF PROTEIN PURITY AND AFFINITY CONTROLS .....	13
AGGREGATION STUDIES .....	13
<b>PROTOCOLS</b> .....	<b>14</b>
FLOW-CYTOMETRIC ANALYSIS; BINDING TO AMYLOID BETA PEPTIDES .....	14
FLOW-CYTOMETRIC ANALYSIS; OFF-RATE ANALYSIS .....	14
FLOW-CYTOMETRIC ANALYSIS; OFF-RATE GATING AND CALCULATIONS .....	14
POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION .....	15
ENZYMATIC DIGESTION .....	15
DEPHOSPHORYLATION OF PET26B+ .....	15
GELEXTRACTION ON A 1 % AGAROSE GEL .....	15
LIGATION .....	15
TRANSFORMATION TO CHEMICAL COMPETENT CELLS .....	15
PCR SCREEN .....	15
ETHANOL PRECIPITATION .....	16
CYCLE SEQUENCING .....	16
HARVESTING PROTEIN FROM <i>E. COLI</i> BL21 CELLS, NATIVE CONDITIONS .....	16
HARVESTING PROTEIN FROM <i>E. COLI</i> BL21 CELLS, DENATURING CONDITIONS .....	16
PREPARATION OF IMAC COLUMNS .....	16
IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC) ASSAY, NATIVE CONDITIONS .....	16
IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC) ASSAY, DENATURING CONDITIONS .....	17
BUFFER EXCHANGE BY PD-10 PREPACKED DESALTING COLUMNS .....	17
SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) .....	17
CONCENTRATING THE SAMPLES .....	17
SIZE EXCLUSION CHROMATOGRAPHY (SEC) .....	17
ELECTROSPRAY IONIZATION TIME OF FLIGHT MASS SPECTROMETRY (ESI-TOF-MS) .....	17
PROTEIN INTERACTION ASSAY (SPR) .....	17
THIOFLAVIN T ASSAY .....	18
<b>RESULTS</b> .....	<b>19</b>
FLOW-CYTOMETRIC ANALYSIS; BINDING OF AMYLOID BETA PEPTIDES .....	19
FLOW-CYTOMETRIC ANALYSIS; OFF-RATE ON STAPHYLOCOCCUS CELL SURFACE .....	19
EVALUATION OF PURITY OF ZABETAMATLIB CONSTRUCTS .....	22

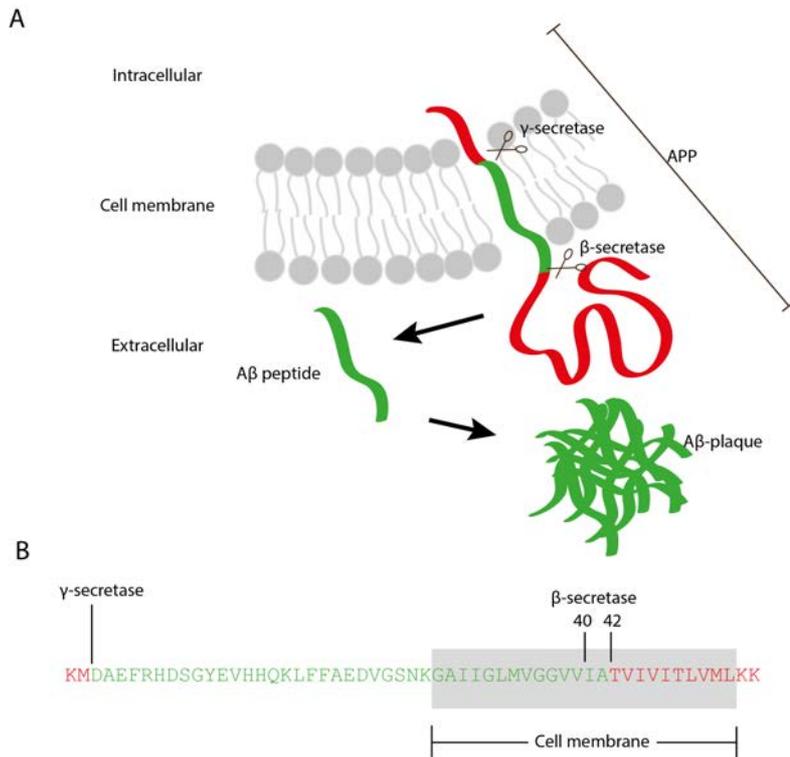
PROTEIN INTERACTION STUDY AND KINETICS .....	24
AGGREGATION ASSAY .....	25
<b>DISCUSSION.....</b>	<b>27</b>
FLOW-CYTOMETRIC STUDIES .....	27
PRODUCTION OF AMYLOID BETA BINDING AFFIBODY MOLECULES .....	27
VERIFICATION OF SIZE.....	29
PROTEIN INTERACTION STUDIES .....	29
AGGREGATION STUDIES .....	30
COMPARING OFF-RATE ON STAPHYLOCOCCUS SURFACE AND IN FREE FORM .....	30
<b>CONCLUSIONS.....</b>	<b>31</b>
<b>ACKNOWLEDGEMENT .....</b>	<b>31</b>
<b>REFERENCE: .....</b>	<b>32</b>
<b>APPENDIX 1. ....</b>	<b>34</b>
<b>APPENDIX 2. ....</b>	<b>35</b>

## Introduction

### Alzheimer's disease

Alzheimer's disease (AD) is the most common type of dementia and accounts for 60 to 80 % of all the dementia cases (Alzheimer's Association, 2009). In 2004, 18 million people suffered from AD and the prognosis does not look good. WHO estimates that this number will double til 2025 (World Health Organisation, 2004). The main cause is not yet established but there are two major diagnostic features, senile plaques and neurofibrillary tangles in the brain, which thereby characterizes AD. Neurofibrillary tangles are associated with the microtubule-protein tau. Tau aggregate and get hyperphosphorylated and cluster inside the cell (Delacourte and Defossez 1986). The main research area today revolves however around the 'amyloid cascade hypothesis'. This hypothesis states that Amyloid- $\beta$  ( $A\beta$ ) peptides, which derive from a large transmembrane protein, Amyloid precursor protein (APP), have a critical role in the development of AD (Hardy and Higgins, 1992). The gene for this protein is located on chromosome 21 and AD was associated with trisomy 21 for a long time, the protein is located on the neurons. APP is expressed constitutively and in high levels not only in the brain but also in other parts of the human body. The main function of this is not known but one theory is that APP is involved in the regulation of synapse formation (Priller *et al.* 2006). The theory states that deposition of  $A\beta$ -peptides is the causative agent and the neurofibrillary tangles together with cell death and dementia is an effect of this (Hardy and Higgins, 1992). Further, it has been shown in several studies that  $A\beta$ -peptides are toxic *in vitro* and cells die as a consequence of this (Korotzer *et al.* 1993, Pike *et al.* 1993).

APP is cleaved into monomers by  $\gamma$ -secretase and  $\beta$ -secretase, and they have a tendency to stick together as dimers. These in turn aggregate and create senile plaques or  $A\beta$ -plaques (Figure 1A). There are several cleavage patterns of APP and these generate different lengths of the  $A\beta$ -peptide. The variant that is coupled with AD is the so-called  $A\beta_{1-42}$  and is 42 amino acids long compared to the predominating peptide,  $A\beta_{1-40}$ . These two have similar chemical properties and does not differ much, but the aggregation rate into plaques are different. The main reason for this is not known but it has been suggested that the C-terminal of the  $A\beta$ -peptide is more structured in  $A\beta_{1-42}$  and is therefor more prone to aggregate (Lim *et al.* 2007, Sgourakis *et al.* 2007). APP is also prone to aggregate into dimers and one of the theories behind this dimerization is that APP acts as a form of receptor in order to bind extracellular proteins, such as heparin and collagen (Beher *et al.* 1996). The dimerization is thought to begin in the N-terminus and as a zipper connect to at least three interaction sites, one of them is in the transmembrane sequence (Kaden *et al.* 2008). The  $A\beta$  peptide contains both the N-terminus juxtamembrane and the transmembrane sequence (Figure 1B).



**Figure 1. Neuron with APP.** Amyloid Precursor Protein is cleaved by  $\gamma$ - and  $\beta$ -secretase, A $\beta$ -peptides is formed which in turn aggregates into A $\beta$ -plaques. (A) Schematic of cleavage. (B) Cleave sites of  $\gamma$ - and  $\beta$ -secretase. Grey area represents the transmembrane region.

### Current and future treatment strategies

There is neither a cure for AD nor a stop of the progression but rather substances that can reduce the symptoms nowadays. In 2012 there were over 300 clinical trials on-going to understand and trying to prevent Alzheimer's disease, 30 of them was in phase III which is the last step before approval and marketing (U.S National Institute of Health, 2012). Today there are two types of medicine that can be administrated to Alzheimer's patients and are approved by the U.S Drug and Food Administration. One type is the acetylcholinesterase inhibitor. Acetylcholinesterase is an enzyme that breaks down Acetylcholine, which leads to increased levels of the neurotransmitter acetylcholine (Alzheimer's Association, 2012). The other type of drug is a NMDA (N-methyl-D-aspartate) receptor antagonist, which is commonly used as anesthesia. The antagonist blocks the NMDA receptor but do not activate it. The blockage inhibits the overstimulation of the glutamate receptor, which is common in AD (Danysz and Parsons 2003). One of the new strategies is to target the  $\gamma$ - and  $\beta$ -secretase. However, this approach has mixed results since the natural occurring enzymes have other functions than just the cleavage of APP. The results show that the inhibition interferes with other important pathways, such as the Notch signaling pathway (Geling *et al.* 2002). Another approach is active immunization against A $\beta$ -peptides. One phase II trial showed promising results till 18 of the 300 patients got severe brain inflammation and the trial had to be stopped (Gillman *et al.* 2005). The most recent strategy is to target the A $\beta$ - peptide directly with monoclonal antibodies, also known as passive immunization. One of the most promising candidates was called Bapineuzumab and is a humanized mouse

antibody and targets A $\beta$  (Salloway *et al.* 2009). Nonetheless, also this trial was canceled by the provider Pfizer Inc. and Johnson & Johnson due to negative results. Alas, the immunization did not work and there was no difference between the immunized patients and the placebo patients (Johnson & Johnson, 2012).

### **Affibody molecule**

Affibody molecules are small, 58 amino acids long affinity proteins that were developed by Nord *et al.* (1997) and originates from the Staphylococcal surface Protein A (SPA). In its native form this protein has affinity for the Fc-part of IgG (Uhlen *et al.* 1983). The B-domain of SPA was isolated and engineered and is now called “Z” and it still had affinity towards IgG. This novel three-alpha helix bundle was proteolytic stable and is one of the fastest refolding protein there is (Myers and Oas, 2001). This cysteine-free affinity protein has been used as a scaffold for building combinatorial bacterial and phage libraries. By using phage-display or staphylococcus display together with different target molecules Affibody molecules can be selected.

By randomizing 13 of the surface amino acids, on two of the alpha helices, the affinity towards IgG was lost but it gained affinity for other target proteins such as HER2, HER3, EGFR and A $\beta$ -peptides in the presence of the ligand. The third helix was kept for stabilization of the affinity protein. This affinity can be used in therapeutic settings (Li *et al.* 2010) as well as in imaging of specific biomarkers (Lyakhov *et al.* 2012). Affibody molecules do not only differ from antibodies by size, Affibody molecules are much smaller, but also by structure since these molecules generally lacks disulphide bridges and consists of alpha helices. One of the advantages of this small affinity protein is that it may be able to penetrate the blood-brain-barrier and thereby is able to target proteins in the brain, e.g. A $\beta$ -peptides.

### **Amyloid beta targeted Affibody molecule**

The small size of this affinity protein is practical when targeting proteins that are in excess in the brain, since it may penetrate the blood-brain-barrier. One of those peptides is the A $\beta$ -peptide. The main goal for creating an Affibody molecule towards the Amyloid beta peptide is that it first should bind to the peptide but then also drain the brain of the peptides by using the peripheral sink mechanism. By altering the equilibrium between the plasma and the central nervous system (CNS) in regards to Amyloid beta the peptides should in theory exit the CNS and be cleared from the brain. Studies with antibodies have shown that this approach actually decreases the A $\beta$  concentration in the CNS in mouse (DeMattos *et al.* 2001).

The first Affibody molecule that targeted the Amyloid beta peptide was created by phage-display 2007 by Grönwall and Jonsson with colleagues. A library was made with  $3 \times 10^9$  variants, 192 was sequenced and revealed 44 unique sequences. The sequencing results showed that all of the different variants had one thing in common. At position 28 all had a cysteine. As mentioned earlier the Affibody molecule is cysteine-free but it seemed in these studies that cysteine have an important role in the binding of Amyloid beta peptide (2007). Biosensor studies in the same article showed that two of the candidates (ZAb<sub>1</sub> and ZAb<sub>3</sub>) had very high affinity for A $\beta$ , with a dissociation constant ( $K_D$ ) up to  $2,6 \times 10^{-7}$  and  $3,20 \times 10^{-7}$  M. Further a disulphide linker was introduced between a head-to-tail dimer of Z<sub>A $\beta$ 3</sub> creating (Z<sub>A $\beta$ 3</sub>)<sub>2</sub> (Hoyer *et al.* 2008). The monomeric A $\beta$  binding Affibody molecule, ZAb<sub>3</sub>, together with Amyloid beta peptides show a decrease in plaque formation suggesting that the Affibody molecule inhibits the formation of A $\beta$ -plaques (Hoyer *et al.* 2007). This has also been studied *in vivo* with fruit flies and the

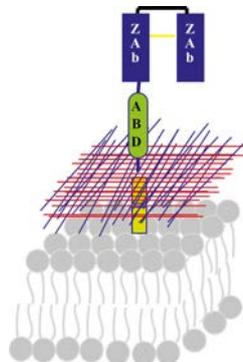
monomeric binder hindered the plaque formation and prolonged the life of the flies (Luheshi *et al.* 2010).

The dimeric Affibody molecule was further modified with the motivation that the N-terminal does not seem to precipitate in the binding according to nuclear magnetic resonance structure. Also the new version was more suited to produce with chemical synthesis. The truncated variants showed lower  $T_m$  values and higher affinity than the un-truncated variant (Lindgren *et al.* 2010).

In 2012 Lindberg *et al.* created a heat-to-tail library based on  $(ZAb_3)_2$  (where cysteine had been excluded) and selected variants with the Staphylococcal cell surface display system. The library consisted of  $10^7$  variants and after five rounds of selection high expression and binding towards  $A\beta$  peptides was isolated. The sorting was done with flow-cytometer. Two of the clones stood out and one peculiar observation was done. Both of the clones had cysteine on both position 28 and position 88. The selection had been made without cysteine but they had been mis-incorporated into the sequence anyway (Lindberg *et al.* 2012) suggesting further that they are important in binding to  $A\beta$ -peptides.

### Staphylococcal cell surface display

Surface display systems have been used since 1980's when George P Smith developed the first phage display system. Here, they fused small proteins and peptides to the phage protein pIII (Smith, 1985). Since the 1980's discovery many different microbial display systems have been engineered, both for Gram-negative and positive bacteria and yeast display. One of the display systems was partly developed in Sweden by Samuelsson with colleagues and they used *Staphylococcus carnosus* and took advantage of the secretion systems in *Staphylococcus hyicus* together with streptococcal protein G. For the first time they could use fluorescence-activated cell sorting methods to analyze fused receptors in gram-positive bacterium. *S. carnosus* do not carries the virulence factors that the *Staphylococcus* genus can be associated with (e.g., toxins, Protein A and haemolysins) so it is relative safe to work with them. By fusing the protein of interest with an Albumin binding domain (Figure 2) both expression and binding can be monitored in different channels in a flow-cytometer. Expression can be observed when adding fluorescently labeled HSA (human serum albumin) and the binding can be observed by labeling of the ligand of interest (Samulesson et al. 1995).



**Figure 2.** Schematic of staphylococcus cell surface display. Cellmembrane in grey with the peptidoglycan layer in red/blue. A head-to-tail dimer of a Affibody molecule is expressed with a linker to a Albumin binding domain.

## Background

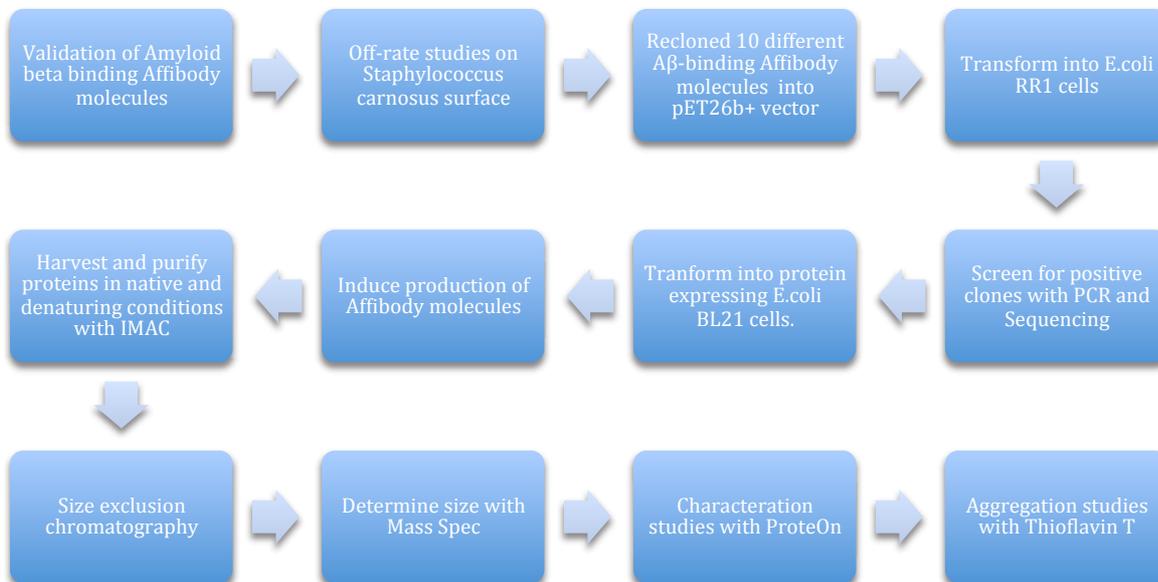
One of the main goals in the biotherapy and bacterial display group at the Royal Institute of Technology is to construct combinatorial libraries of Affibody molecules that have been engineered at this department. This affinity molecule has then been selected towards different targets as previous said. The group has as well developed a novel vector system in order to select for these binders together with flow-cytometer sorting, the staphylococcus surface display system (Kronqvist *et al.* 2008).

The A $\beta$  binding Affibody molecule has been developed since 2007 and several modifications has been made from the original monomeric A $\beta$  binding Affibody molecule in order to increase affinity towards A $\beta$ . The latest thing was to create two maturation libraries with the Affibody were it was head-to-tail engineered and N-terminally truncated in order to get higher affinity and to get a slower off-rate than the original A $\beta$  binding Affibody molecule, Z<sub>A $\beta$ 3</sub>. The selection had then been done with regards to the off-rates by Lindberg (unpublished data) and eight of the variants that showed the slowest off-rates and highest affinity were chosen for further characterization studies.

## Aim

The aim of this project was to produce and characterize 8 Amyloid beta binding Affibody molecules, with two Amyloid beta binding Affibody molecules as controls, from an affinity maturation library aimed for potential therapeutic and diagnostic applications.

## Workflow



## Materials

### Buffers

#### **IMAC wash buffer (Native conditions)**

<b>pH 7.5</b>	
<i>Na<sub>2</sub>HPO<sub>4</sub></i>	20 mM
<i>NaCl</i>	300 mM
<i>Imidazole</i>	15 mM
<i>MQ</i>	

#### **IMAC elution buffer (Native conditions)**

<b>pH 7.5</b>	
<i>Na<sub>2</sub>HPO<sub>4</sub></i>	20 mM
<i>NaCl</i>	300 mM
<i>Imidazole</i>	150 mM
<i>MQ</i>	

#### **IMAC lysis buffer (denaturing conditions)**

<b>pH 8</b>	
<i>Guanidiniumchloride</i>	7M
<i>Na<sub>2</sub>HPO<sub>4</sub></i>	47 mM
<i>NaH<sub>2</sub>PO<sub>4</sub></i>	2.65 mM
<i>Tris-HCl pH8</i>	10 mM
<i>NaCl</i>	100 mM
<i>MQ</i>	

#### **IMAC wash buffer (denaturing conditions)**

<b>pH 8</b>	
<i>Guanidiniumchloride</i>	6 M
<i>Na<sub>2</sub>HPO<sub>4</sub></i>	46.6 mM
<i>NaH<sub>2</sub>PO<sub>4</sub></i>	3.4 mM
<i>NaCl</i>	300 mM
<i>MQ</i>	

#### **IMAC elution buffer (denaturing conditions) pH 5**

<i>Urea</i>	6 M
<i>Na<sub>2</sub>HPO<sub>4</sub></i>	50 mM
<i>NaCl</i>	100 mM
<i>Glacial Acetic acid</i>	30 mM
<i>NaAc</i>	70 mM

#### **10 x PBS**

<i>NaCl</i>	770 mM
<i>NaH<sub>2</sub>PO<sub>4</sub></i>	300 mM
<i>Sterile glucose mix</i>	2 %
<i>MQ</i>	

#### **10 x PBS-P**

<i>10 x PBS</i>	10 %
<i>Pluronic</i>	909µM
<i>MQ</i>	

#### **5 x OX SDS PAGE loading buffer**

<i>Titripex III EDTA</i>	
<i>SDS</i>	433 mM
<i>Tris-HCl</i>	100 mM
<i>Bromophenol blue</i>	5 mM

#### **Sterile glucose mix**

<i>Glucose</i>	50 %
<i>MgSO<sub>4</sub></i>	0.1 mM
<i>Thiamineacid</i>	0.1 mM
<i>CaCl<sub>2</sub></i>	0.1 mM
<i>MQ</i>	

#### **BFB mix**

<i>Concentrated BFB</i>	5 %
<i>Glycerol</i>	30 %
<i>MQ</i>	

#### **TSB + Y**

<i>Tryptic soy broth</i>	30 g
<i>Yeast extract granulate</i>	5 g
<i>MQ</i>	Fill up to 1000 ml

#### **20 x MES**

<i>MES</i>	500 mM
<i>Tris-base</i>	380 mM
<i>SDS</i>	4 mM
<i>EDTA</i>	0.8 mM
<i>MQ</i>	

#### **50 x TAE**

<i>Tris-base</i>	1.15 M
<i>CH<sub>3</sub>CO<sub>2</sub>H</i>	57.1 mM
<i>EDTA</i>	2 mM
<i>MQ</i>	

#### **10 x Cycle sequencing buffer (CS)**

<i>Tris-Base</i>	3 mM
<i>MgCl<sub>2</sub></i>	6.5 mM

#### **SPR regeneration buffer**

0,05% Sodium dodecyl sulphate  
Alt. 0,25% Sodium dodecyl sulphate

#### **SPR running buffer**

<i>HEPES</i>	5 mM
<i>NaCl</i>	150 mM
<i>EDTA</i>	3,4 mM
<i>Tween 20</i>	0,005%
<i>MQ</i>	

#### **5 x RED SDS PAGE loading buffer**

<i>Titripex III EDTA</i>	5 mM
<i>SDS</i>	433 mM
<i>B-mercaptoethanol</i>	3,5 M
<i>Tris-HCl</i>	100 mM
<i>Bromophenol blue</i>	5 mM

## Bacterial strains

In this project I used two different *Escherichia coli* strains in order to produce the ZAbetamatlib binders. One *Staphylococcus* strain was used in all FACS experiments.

*E. coli* RRI ΔM15 (Ruther 1982)

*E. coli* BL21(DE3) (Studier, 1986)

*S. carnosus* TM300 (Augustin and Gotz, 1990)

## Equipment

Instruments

Heating block

SDS gel bath

Spectrophotometer

Gel electrophoresis gel bath

Plate seal

Centrifuge

Centrifuge

Table top centrifuge

Sonicator

PCR machines

Sequencer

Rotamix

ÄKTA Explorer

MS

SPR

Flow-cytometer

French®Pressure cell press

Plate reader

Grant Instruments

Invitrogen Xcell SureLock Electrophoresis cell

Eppendorf BioPhoto meter

BioRad

TechtumLab, ABgene ALPS-300

Heraeus Instruments, Multifuge 3S-R

BECKMAN COULTER, avanti J-26XP centrifuge

Heraeus Instruments, BiofugePico

Sonics, vibra cell

Applied Biosystem, GeneAmp PCR system 9700

Agilent Technologies, 3730XL DNA Analyser

TechtumLab, Intelli-mixer

GE Healthcare Lifescience

Agilent Technologies, 6520 Accurate Mass, Q-TOF LC/MS

Bio Rad, ProteOn™ XPR36

BECKMAN COULTER, Gallios™

SLM Instruments Inc.

Tecan Trading AG

## Software

Excel (2011)

Word (2011)

Power Point (2011)

Illustrator (CS6)

Masshunter Qualitative Analysis

Kaluza Flow-cytometry analysis (v.1.1)

Unicorn (5.11)

Geneious (R6)

i-control

ProteOn Manager Software

Microsoft Office

Microsoft Office

Microsoft Office

Adobe

Agilent Technologies

Beckman coulter group

GE Healthcare Lifescience

Biomatters Ltd.

Tecan trading AG

BioRad

## Methods

### Staphylococcus display

The affinity towards A $\beta$ -peptides was validated in a flow-cytometer in a Staphylococcus display system. The ZAbetamatlib constructs were displayed on the surface of *Staphylococcus carnosus* and incubated with biotinylated A $\beta$ -peptides. The cells were then incubated with fluorescently labeled streptavidin and human serum albumin (HSA). The streptavidin has high affinity towards biotin and HSA has affinity towards the albumin binding domain (ABD) that is expressed together with ZAbetamatlib constructs (Figure 2). So, in the flow-cytometer two channels were used, FL-2 which detect the streptavidin and thereby the binding towards A $\beta$  and the FL-6 channel we can detect HSA and hence the expression levels. When combining these two channels we can see if the Affibody molecule is expressed and is binding the target. When the affinity was validated an off-rate study was done. In this study we wanted to see the release of the A $\beta$ -peptides. So, first the cells were incubated with biotinylated amyloid beta, then after 45 minutes they were incubated with a molar excess of non-labeled Amyloid beta. In theory, when the labeled A $\beta$  is released from the A $\beta$  binding Affibody molecule a non-labeled A $\beta$  will take its place and we will see a reduction in the binding, the FL-2 channel.

### Isolation and purification of Zabetamatlib constructs

Nine constructs were isolated from a pScZ1 vector (Kronquist *et al.* 2008), one construct was ordered as a gene from geneScript US Inc. and isolation was not necessary, with primers (table 1) containing restriction sites for the enzymes NdeI and XhoI by a polymerase chain reaction (PCR). A PCR purification was performed using QIAquick PCR purification kit. All constructs together with a pET26b+ vector were digested with previous mentioned enzymes according NEB's recommendations and the vector was then dephosphorylated as mentioned in the procedures section. In order to remove potential monomers an isolation was performed on all the constructs and the vector by gel extraction and QIAquick Gel Extraction Kit according to their instructions. The constructs were then ligated to the pET26b+ vector with T4 DNA ligase. The gene that was ordered was later isolated from the pET26b+ vector in order to ligate it into the pScZ1 vector. Two of the constructs were later isolated with primers (and cleaved with NcoI) that overlapped the PelB sequence in the pET26b+ vector.

**Table 1.** Primers used in cloning and sequencing of ZAbetamatlib constructs

Name	Sequence	Description
LIHA-52	TGCGCAGGATCCCCATATGGCGGGTGGGAGAT	Forward primer containing Nde1 restriction site. Used with construct (ZAb3A12) <sub>2</sub> VE
LIHA-53	TGCGCAGGATCCCCATATGGCGGGTCGCGA	Forward primer containing Nde1 restriction site. Used with construct ZAbetamatlibSYM#25
LIHA-54	TGCGCAGGATCCCCATATGGCGGGTGGCGAA	Forward primer containing Nde1 restriction site. Used with construct ZAbetamatlibSYM#57, ZAbetamatlibSYM#73, ZAbetamatlibAS#33
LIHA-55	TGCGCAGGATCCCCATATGGCGGGTGGCGAGT	Forward primer containing Nde1 restriction site. Used with construct ZAbetamatlibAS#56, ZAbetamatlibAS#66, ZAbetamatlibAS50nM#76
LIHA-56	TGCGCAGGATCCCCATATGGCGGGTGGCGAACGT	Forward primer containing Nde1 restriction site. Used with construct ZAbetamatlibAS50nM#64
LIHA-57	GCATGCCTGCAGCTCGAGTTTCGGCGCCTGAGCAT	Reverse primer containing Xho1 restriction site. Used on all constructs.
LIHA-66	ATACATCTCGAGGCGGGTGGGAGAT	Forward primer containing Xho1 restriction site. Used with construct (ZAb3A12) <sub>2</sub> SG4. Amplification from pET26b+
LIHA-67	TATGTGGCTAGCAAGTTAGCGCTTTGGCTTG	Reverse primer containing Nhe1 restriction site. Used with (ZAb3A12) <sub>2</sub> (S4G) <sub>2</sub> . Amplification from pET26b+
LIHA-68	TATGTGCCATGGCGGGTGGCGAACGC	Forward primer containing Nco1 restriction site. Used for ZAbetamatlibSYM#57 and#73
T7 fwd	TAATACGACTCACTATAGGG	Forward primer that anneals to pET26b+ vector in front of ZAbetamatlib construct
T7 rev	GCTAGTTATTGCTCAGCGG	Reverse primer that anneals pET26b+ after the ZAbetamatlib construct.
SAPA 23	GGCTCCTAA AGAAAATACAACGGC	Forward primer that anneals to pScZ vector in front of ZAbetamatlib construct
SAPA 24	TGTTGAATTCCTTAAGGGCATCTGC	Reverse primer that anneals pScZ1 after the ZAbetamatlib construct.

### Insertion and expression of vectors

Each vector containing the construct was transformed into *E. coli* RR1ΔM15 (Rüther, 1982) cells by heat-shock. In order to determine whether the cells contained the different ZAbetamatlib constructs a PCR screen was performed and controlled on a 1% agarose gel. PCR products that showed the expected size on the agarose gel was then precipitated with ethanol and sequenced in order to confirm the right sequence. Ten different colonies containing the ten different ZAbetamatlib constructs were then incubated in 10 μl tryptic soy broth + yeast extract (TSB + Y) with 50 μg/μl Kanamycin in 37 °C over night. The plasmids was purified with QIAprep Spin

Miniprep Kit to isolate the plasmid and transformed to protein expressing *E.coli* BL21(DE3) (Studier, 1986) cells and the plates together with 50 µg/ µl was left in 37 °C over night. One colony containing each construct were inoculated in 200 µl TSB + Y together with 50 µg/ µl kanamycin in 37 °C with 150 rpm shaking with the final OD of 0.07. When OD600 reached 0,7 the inoculate was induced with 0,5 M IPTG. This was set to incubate over night in 25 °C, 200 rpm shaking. The cells were then harvested by centrifugation at 4000 rpm in 8 minutes. The supernatant were thrown away and the pellet was set to incubate in -20 °C for 10 minutes. The cells were then lysed with different methods depending on IMAC purification conditions.

### **Purification with IMAC and SEC**

All constructs were IMAC purified with NAP-10 columns with Cobalt HisPur affinity resin (Thermo Scientific). The buffer was then changed by PD-10 desalting columns to 1 x PBS. The presence of proteins of the right size was controlled on a SDS-page gel using both oxidative and reducing conditions to see if there was potential multimers. The concentration of the different proteins was determined with absorbance at 280 nm and calculated from their molecular weight and extinction coefficient with Beer–Lambert law. All constructs were concentrated with a vivaspin 2 ml and the constructs were purified by size exclusion chromatography on a ÄKTA explorer 10. In order to determined the concentration of the purified proteins a BCA analysis was preformed.

### **Verification of protein purity and affinity controls**

All constructs were analyzed with Electrospray analyzing time of flight mass spectrometry (ESI-TOF-MS) in order to confirm the calculated molecular weight from ExPASy ProtParam.

### **Aggregation studies**

As the original binder inhibits plaque formation in vivo (Luheshi *et al.* 2010) we want to investigate whether these constructs inhibit the formation as well. This is done with a florescent molecule, Thioflavin T (ThT). ThT is used to monitor the amyloid beta plaque formation since it binds the aggregated A $\beta$  and emits light. This method was used to see if the purified A $\beta$  binding Affibody molecules prevent the aggregation of amyloid beta. So ThT was incubated with A $\beta$ <sub>1-42</sub>-peptides and the Affibody molecules and the fluorescence was measured every 15 minutes for 20 hours in a plate reader.

## Protocols

### Flow-cytometric analysis; binding to Amyloid beta peptides

All ZAbetamatlib constructs in *S. carnosus* together with Zwt, which has affinity for IgG, was inoculated in 10 mL TSB + Y with 10 µg/mL CML at 37°C over night. 10 µL of the over night cultures was centrifuged with 800 µL PBS-P at 6000 rpm, 4°C for 6 minutes in Eppendorf tubes. The supernatant was thrown away and the cell pellet was then washed 2 times with 200 µL PBS-P with centrifugation. The cell pellet was then resuspended with sonicated 50 µL 10 nM N-biotinylated Aβ-peptides (Zwt with 0,22 µM biotinylated IgG) and incubated for 45 minutes in room temperature in a rotamixer. The suspension was then centrifuged at 6000 rpm for 6 minutes in 4°C and washed once with 200 µL PBS-P. The cell pellet was resuspended with 200 µL of HSA conjugated with ALEXA 647 (Invitrogen) and 200 µg/mL Streptavidin conjugated with R-phycoerythrin 488 nm (Invitrogen) in PBS-P. This was set to incubate in dark, on ice for 15 minutes. Lastly the cells was washed two times and then transferred into 5 mL round-bottomed Falcon tubes and analysed on Gallios™ flow-cytometer from Beckman Coulter.

### Flow-cytometric analysis; Off-rate analysis

All ZAbetamatlib containing *Staphylococcal carnosus* variants was inoculated in 10 mL TSB + Y with 10 µg/mL CML at 37°C over night. 100 µL of the overnight cultures together with 800 µL PBS-P was centrifuged at 6000 rpm, 4°C for 6 minutes in Eppendorf tubes. The supernatant was thrown away and the cell pellet was washed two times in 800 µL PBS-P. After the washes the pellet was resuspended in 50 nM sonicated N-biotinylated Aβ-peptides together with HSA conjugated with ALEXA 647 (Invitrogen) and incubated for 45 minutes in a rotamixer at room temperature. After the incubation the cells with Aβ-peptides was centrifuged at 6000 rpm, 4°C for 6 minutes. The supernatant was thrown away and the pellet was washed one time with 800 µL PBS-P. The pellet was then resuspended with 300 µL 100nM sonicated unlabelled Aβ-peptides. Each time point (0, 1h, 2h, 3h, 4h, 5h, 6h) 30 µL of the mixture was collected and centrifuged at 6000 rpm, 4°C for 2 minutes and the pellet was washed one time with 200 µL PBS-P. The pellet was then resuspended in 80 µL 200 µg/mL Streptavidin conjugated with 200 µg/mL R-phycoerythrin 488 nm (Invitrogen) in PBS-P for 15 minutes. After 15 minutes the cells was centrifuged at 6000 rpm, 4°C for 2 minutes and the pellet was washed one time with 200 µL PBS-P. The cell pellet was then resuspended in 200 µL PBS-P and transferred into 5 mL round-bottomed Falcon tubes and 20 000 events was analysed on Gallios™ flow-cytometer from Beckman Coulter. This analysis was done in two replicates in two separate days.

### Flow-cytometric analysis; Off-rate gating and calculations

In order to do a proper calculation a gate around the expressing and binding population was done. All unspecific binding was ungated. This gate was then transferred into the one dimension FL-2 histogram. All time points from each construct were overlaid and the X-median value was noted. This X-median was then plotted against time and a non-linear regression was made. The off-rate was calculated with GraphPad Prism 6 (GraphPad software Inc.) (Equation 1).

$$(1) \quad Y = (Y_0 - Plateau) * \exp(-k * X) + Plateau$$

In equation 1 Y is the X-median value contained from flow-cytometric analysis, Y<sub>0</sub> is the median value at time=0 seconds, the plateau is non-bound Aβ and X is the time points in seconds. k is the k<sub>d</sub>-value that we are interested in. All k<sub>d</sub>-values came two replicates and the mean value was calculated.

### **Polymerase chain reaction (PCR) amplification**

A PCR amplification was done on all constructs, which was in the pScZ1 vector in *Staphylococcus carnosus* (not (ZAb3A12)2 (S4G)2). Each colony was picked and dipped in 10 µl milli-q water. From each colony with water mixture 1 µl was transferred into a new PCR tube. Each PCR-tube contained 2mM dNTPs, 1 µM forward primer, 1 µM reverse primer (Table 1), 5xGC buffer (Finnzymes) and Phusion polymerase (Finnzymes). All the reactions together with a negative control (without template) was put in a thermocycler (GeneAmp PCR system 9700) with the following program: 98 °C for 3 minutes; 98 °C for 10 seconds, 68 °C for 30 seconds and 72 °C for 30 seconds, this was repeated 30 times, 72 °C for 2 minutes and lastly 4 °C.

### **Enzymatic digestion**

Restriction enzymes from New England Biolabs were used in this project. One standard unit per µg DNA was used together with 10x NEB4 buffer, also provided by New England Biolabs. First Nde1 was added to the DNA together with the buffer and were set to incubate for 1 hour, 37 °C. Then 100x BSA was added together with Xho1, this was set to incubate for an additional hour in 37 °C. After total 2 hours the digestion was heat-inactivated at 65 °C, 10 minutes. The construct that was cloned into the pScZ1 vector was cleaved with Nhe1 and Xho1. The two constructs that were going to be linked to pelB in the pET26b+ vector were cleaved with Nco1 and Xho1 in the same manner as described.

### **Dephosphorylation of pET26b+**

Antarctic Phosphatase together with 10x Antarctic Phosphatase buffer from New England Biolabs with Milli-q H<sub>2</sub>O was added to the pET26b+ vector and was set to incubate for 1 hour, 37 °C. Heat-inactivation was performed at 65 °C for 10 minutes.

### **Gelextraction on a 1 % Agarose gel**

The DNA was isolated using prestained EtBr 1 % Agarose gel. The right length was observed and excised from the gel using a scalpel. The DNA was then purified by QIAGEN gel purification kit according to manufacturers instructions.

### **Ligation**

All constructs were ligated to the pET26b+ vector by Vector 1:3 Insert. 10x T4 DNA ligase buffer together with T4 DNA ligase provided by New England Biolabs was used. The mixture were set to incubate for 2 hour at RT then heat-inactivated at 60 °C for 20 minutes. Ligation into pScZ1 vector was done in the same manner.

### **Transformation to chemical competent cells**

Transformation into both *E.coli* BL21 and RR1 was done by heat-shock. 800 ng plasmid was added to 100 ml cells and was set to incubate for 30 minutes on ice. The mixture was then heated for 45 seconds in 42 °C and put on ice for 5 minutes. 100ml TSB+Y was added to the cells and they were put in a rotamix in 37 °C for 30 minutes. After incubation the cells were streaked on TBAB plates containing kanamycin and was left for incubation in 37 °C over night.

### **PCR screen**

A polymerase chain reaction was performed in order to see if the transformed RR1 cells had the right insert. Approximately 30 colonies from each construct was picked and dipped in 10 µl milli-Q water. From each well 1 µl was transferred into a new 96-well PCR plate. The wells also contained 200 µM dNTPs, 10x optimized DyNAzyme buffer (Finnzymes), 0,01 U/ µl DyNAzyme II DNA polymerase (Finnzymes), milli-Q water and 0,5 µM T7 forward and reverse primer. A multistep thermocycling protocol was performed in GeneAmp PCR system 9700 with the following steps: 94 °C for 10 minutes; 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for two minutes x2 cycles; 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for two

minutes x2 cycles; 94 °C for 30 seconds, 47 °C for 30 seconds and 72 °C for 2 minutes x 30 cycles; 72 °C for 10 minutes and lastly 4 °C.

### **Ethanol precipitation**

Both the PCR screen and the cycle sequencing DNA were precipitated with ethanol. 10 µl PCR products or cycle sequencing products were added to ice cold 96 % ethanol (2,5 x DNA volume) with 3M NaAc pH 5,5 (0,1 x DNA volume) and was incubated in -20 °C for 20 minutes. The 96 well plates were then centrifuged in 20 minutes at 4000 x g. The supernatant were thrown away and the pellet was washed with ice cold 70 % ethanol and centrifuged for additional 15 minutes. The supernatant were thrown away and the pellet was resuspended in 10 µl milli-q water.

### **Cycle sequencing**

The ethanol precipitated PCR screen and amplified DNA was sequenced by a cycle sequencing reaction. 1 µl DNA was mixed with 5 µg/µl T7 primer (one reaction in each direction), 6 µl 1 xCS buffer and 1 µl BigDye terminator mix (Applied Biosystems). The reaction was extended in GeneAmp PCR system 9700 (Applied Biosystems) using the standard protocol: 96 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 4 minutes in 30 cycles. The sequencing reaction was precipitated with ethanol and resuspended in 10 µl milli-q water. The sequencing was done in 3730xl DNA analyser (AME bioscience).

### **Harvesting protein from *E.coli* BL21 cells, native conditions**

The centrifuged cells were stored in -20 °C for 10 minutes and resuspended in 10 ml IMAC wash buffer (native conditions) and sonicated by vibra cell (sonics) with an amplitude of 23 %, 1.0 seconds pulse, 1.0 seconds pause for 1,5 minutes on ice. The sample was then cooled on ice for 1.5 minutes and previous scheme was used an additional time. The sonicated cells were then centrifuged at 25 000 x g for 15 minutes in 8 °C. The supernatant was collected and filtered through a 0.45 µm filter. The lysate were stored on ice until loading on the IMAC column.

Alt. the cells was resuspended in 10 ml/g pellet and French-pressed (SLM instruments Inc.) in three cycles.

### **Harvesting protein from *E.coli* BL21 cells, denaturing conditions**

The centrifuged cells were stored in -20 °C and resuspended in 10 ml IMAC lysis buffer (denaturing conditions), vortexed and incubated in 37 °C on 150 rpm shaking for 2 hours. The lysis mix were then centrifuged at 16 000 rpm for 20 minutes at 4 °C and the supernatant were collected. The supernatant were filtered through a 1,2 µm filter first then a 0,45 µm filter. The lysate were stored on ice until loading on IMAC column.

### **Preparation of IMAC columns**

Empty PD-10 columns were packed with cobalt HisPure Affinity resin (Thermo scientific) in order to purify the proteins. A filter was placed in the bottom of the column, 2 ml matrix was added and finally a filter on top. The matrix were pulsed with milli-Q water and stored in 4 °C.

### **Immobilized metal ion affinity chromatography (IMAC) assay, native conditions**

The matrix was pulsed with 20 ml milli-q water and equilibrated with 10 CV wash buffer (native conditions). The sonicated or French-pressed lysate was added and washed with 50 ml IMAC wash buffer (native conditions), everything was collected in order to control the steps on a SDS-page gel later. The proteins were eluted in 10 x 1 ml fractions with IMAC elution buffer (native conditions). The fractions were measured at A<sub>280</sub> and fractions with the highest absorbance were pooled.

### **Immobilized metal ion affinity chromatography (IMAC) assay, denaturing conditions**

The matrix was pulsed with 2,5 CV IMAC lysis buffer (denaturing conditions) and 2,5 CV IMAC elution buffer (denaturing conditions) twice and equilibrated with 2,5 CV IMAC lysis buffer (denaturing conditions). The lysate was added and re-loaded after flow-through and washed with 15 ml IMAC wash buffer (denaturing conditions), everything was collected and controlled on SDS-page gel. The protein was eluted in 10 x 1 ml fractions with IMAC elution buffer (denaturing conditions). The fractions were measured at  $A_{280}$  and fractions with the highest absorbance were pooled.

### **Buffer exchange by PD-10 prepacked desalting columns**

In order to store the proteins, the buffer had to be changed into something that do not harm the proteins, 1 x PBS. This was done with a PD-10 desalting column from GE healthcare according to manufactures instructions.

### **Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-page)**

To see if the purification was successful and the right proteins were present a SDS-page gel was performed. The samples were prepared under both reducing and oxidizing conditions to see potential multimers. 1  $\mu$ g protein was prepared with 5  $\mu$ l Red/ox, 2  $\mu$ l 87 % glycerol and milli-q water to a final volume of 20  $\mu$ l. This was heated at 95 °C for 5 minutes and loaded on a NuPage 4 –12 % bis-tris gel (Invitrogen) together with a low molecular weight ladder from GE healthcare that was located in a gel-bath with 1x MES. The gel ran at 200 V for 45 minutes in 4 °C. The gel was then stained with 50 ml GelCode Blue (Thermo Scientific) over night and wash with water for at least an hour.

### **Concentrating the samples**

In order to perform a more time effective size exclusion chromatography the samples were concentrated using Vivaspinn 2 ml, 3000 MWCO PES from Satorius stedim biotech in Heraeus Multifuge 3S-R following manufactures recommendations.

### **Size Exclusion Chromatography (SEC)**

To purify the samples further and sort out potential monomers a size exclusion chromatography was performed on a ÄKTA explorer 10 (GE healthcare) with a Superdex 75 column from GE healthcare. The column volume (CV) was 21 ml. Between each construct the column was washed with 2 CVs of 1 x PBS and between each sample within a construct the column was washed with 1 CV of 1 x PBS. Each fraction size was 1 ml.

### **Electrospray ionization time of flight mass spectrometry (ESI-TOF-MS)**

2  $\mu$ l with the concentration of 0,5  $\mu$ M (calculated with the molecular weight from ProtParam) of each construct together with 18  $\mu$ l of 5 % of ACN 0.01 % FA was tested in the mass-spectrometer, 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies) according to manufactures recommendations. By deconvoluting the results with Masshunter qualitative analysis (Agilent technologies) molecular weights was obtained.

### **Protein interaction assay (SPR)**

The interaction and the binding of the ZAbetamatlib proteins to Amyloid beta peptides was analyzed on a Biorad ProteOn XPR36 C-terminal biotinylated and N-terminal biotinylated A $\beta$ -peptides was immobilized on a NLC-sensor chip (BioRad). The N-terminal biotinylated A $\beta$  was immobilized to a level of 50 RU (Response Units), 500 RU and 1400 RU, the C-terminal variant was immobilized to 500 RU and 800 RU at 22° C. 50 nM, 25 nM, 12,5 nM and 6,25 nM of all ZAbetamatlib proteins was flown over the chip with the flow rate of 50 ml/min for 300 seconds. The dissociation time was first set to 3600 seconds. The candidates that showed the highest  $k_d$  was the tested with a dissociation time of 7600 seconds. The chip was washed with 3 x 0,05%

SDS. For the second trial the chip was washed with 3 x 0,25% SDS. As a control a previously affinity determined Affibody molecule ( $Z_{A\beta 3}(12-58)$ ) was used (Lindgren *et al.* 2010). The kinetics (Langmuir) was calculated with ProteOn Manager and a  $X^2$  under 10 was accepted.

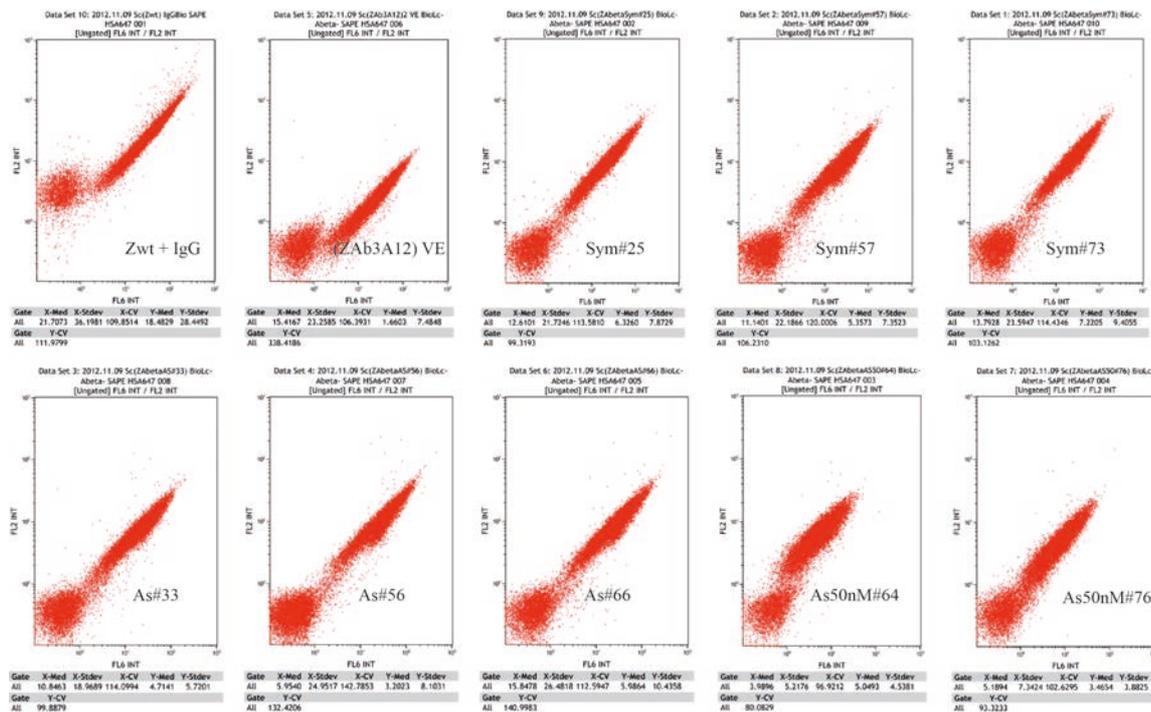
### **Thioflavin T assay**

20  $\mu$ M of Thioflavin T (AnaSpec) was mixed with 30  $\mu$ M  $A\beta_{1-42}$  (or  $A\beta_{1-40}$ ), 33  $\mu$ M ZAbetamatlib constructs and 1 x PBS to the total volume of 50  $\mu$ l. As a positive control ThT together with Amyloid beta peptides and 1 x PBS was used. A negative control was done with an Affibody molecule that targets Prostate-specific membrane antigen (PSMA). The plate was sealed with polyolefin tape and put into a Tecan plate reader. All measurements was done in 37  $^{\circ}$ C and with 10 s orbital shaking before each measurement. Excitation was set to 450 nm and emission at 482 nm (Abs 412 nm). Data was collected every 15 minutes for 20 hours and the data was analyzed in i-control<sup>TM</sup> (Tecan) and Microsoft excel (Microsoft).

## Results

### Flow-cytometric analysis; binding of Amyloid beta peptides

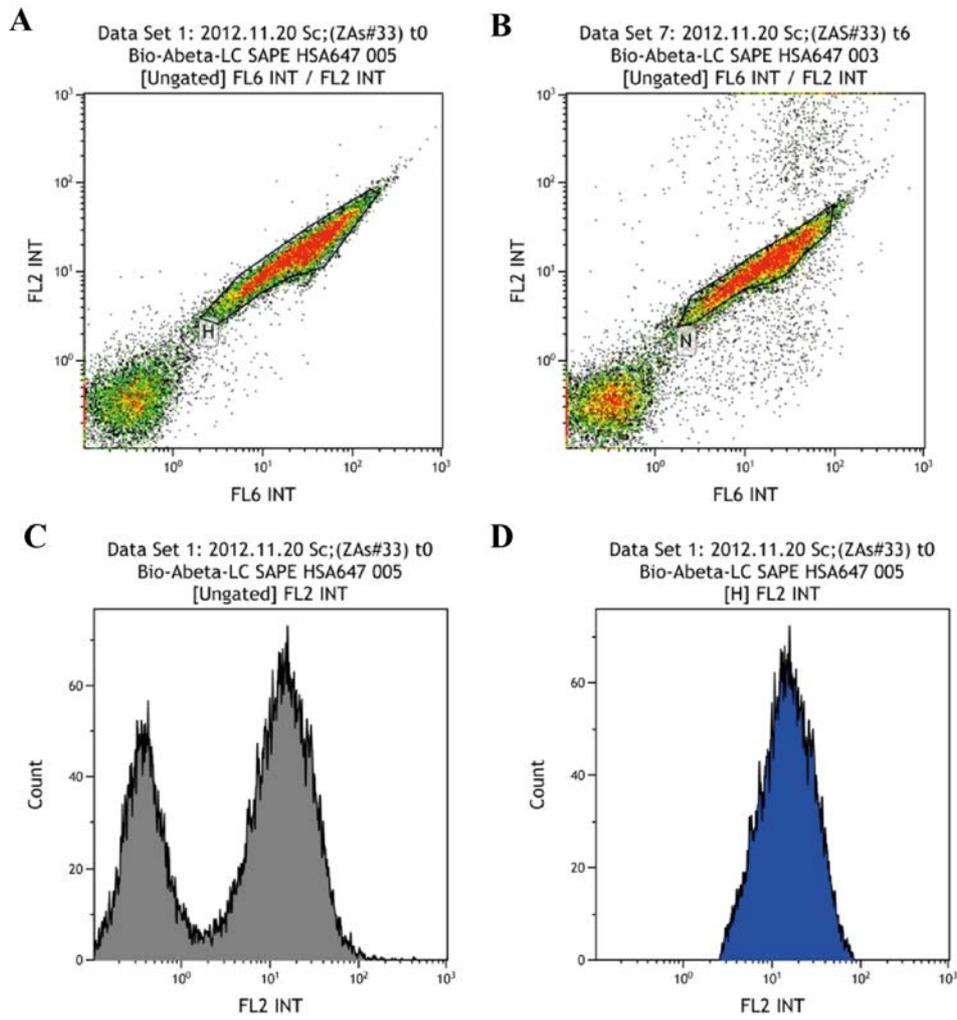
All of the ZAbetamatlib constructs together with one of the dimeric original binders as control, (ZAb3A12)2 VE, together with Zwt showed good binding towards the target, A $\beta$ -peptides (IgG with Zwt), and good expression on the cells (Figure 3). All the constructs had stronger signals than ZAb3A12)2, suggesting a higher affinity.



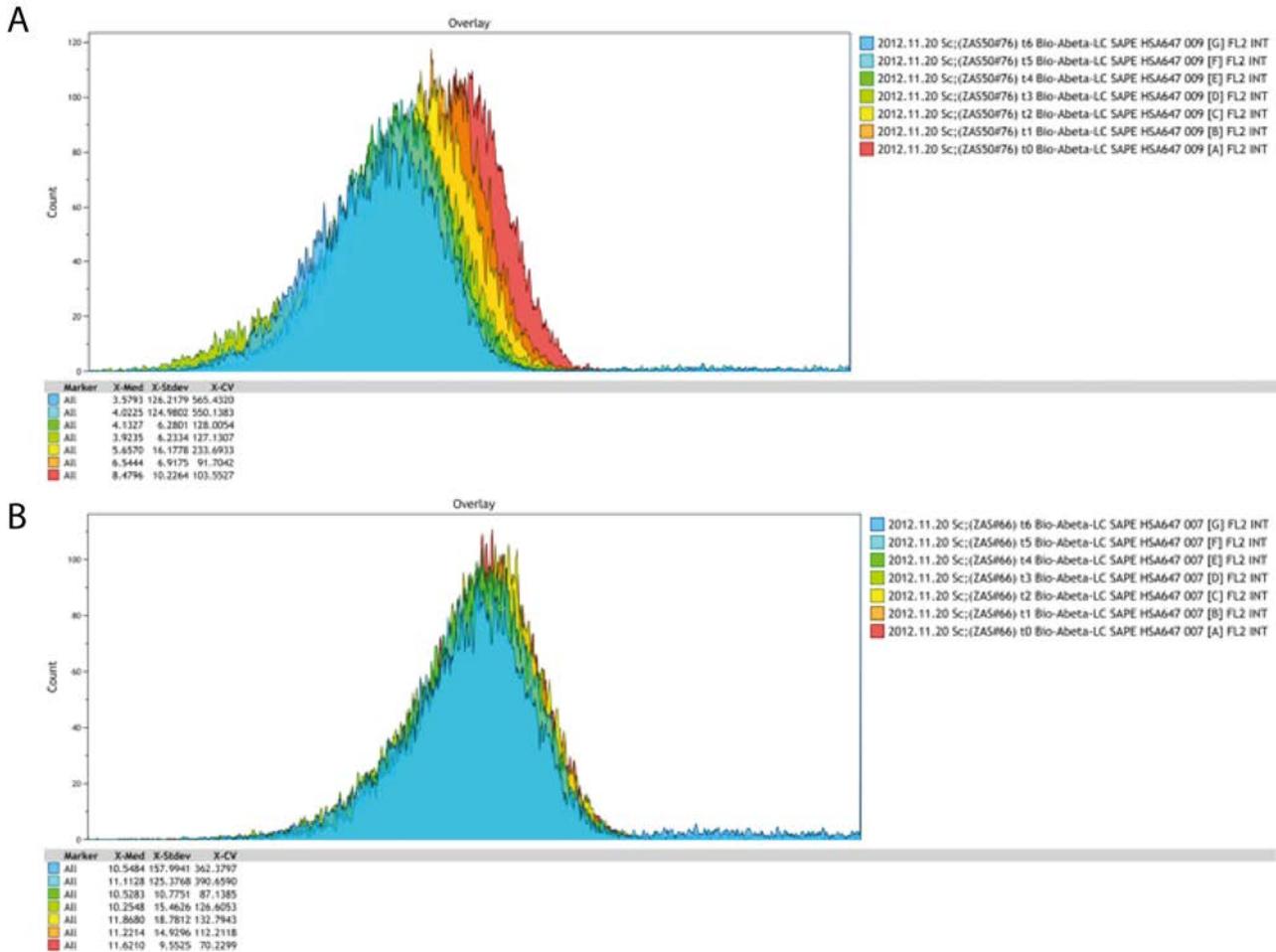
**Figure 3.** Two dimension dotplot where each dot represents an event. X-axis is the FL-6 channel and Y-axis is the FL-2 channel. Expression can be detected in FL-6 and binding in FL-2 channel.

### Flow-cytometric analysis; Off-rate on Staphylococcus cell surface

With gates around the expressing and binding population (Figure 4)  $k_d$ -values could be calculated and an overlay of each time point show good representation of the release of the A $\beta$ -peptide (Figure 5).



**Figure 4.** Gates of flow-cytometric data. (A) Gate around the binding and expressing population at time=0. (B) Gate around the expressing and binding population at time=6h. (C) Non-gated FL-2 histogram showing two populations, one binding to Amyloid beta (middle) and one non-binding population (left). (D) Gate from (A) used in FL-2 histogram, showing only binding population. A representation from all constructs



**Figure 5.** Gates from two dimension dotplots applied on one dimension FL-2 histogram and an overlay of each time point from two constructs. (A) Overlay of AbetamantlibAs50nM#76. Red in back is t=0 and blue in front is t=6h. (B) Overlay of ZAbetamatlibAs#66. Blue in front is t=6. ZAbetamatlibAs#66 appear to have a slower off-rate.

All X-median values that were obtained for each time point and were plotted against time. A curve was created with a non-linear regression fit and off-rates were calculated (Figure 6, Table 2, Appendix 1).  $k_d$ -values differs between constructs and some show an extremely slow off-rate.



**Figure 6.** Two ZAbetamatlib constructs were median value from the flow-cytometric analysis is plotted against time (x-axis) and a non-linear regression fit. As#66 (left) suggest a slower offrate than As50nM#76 (right).

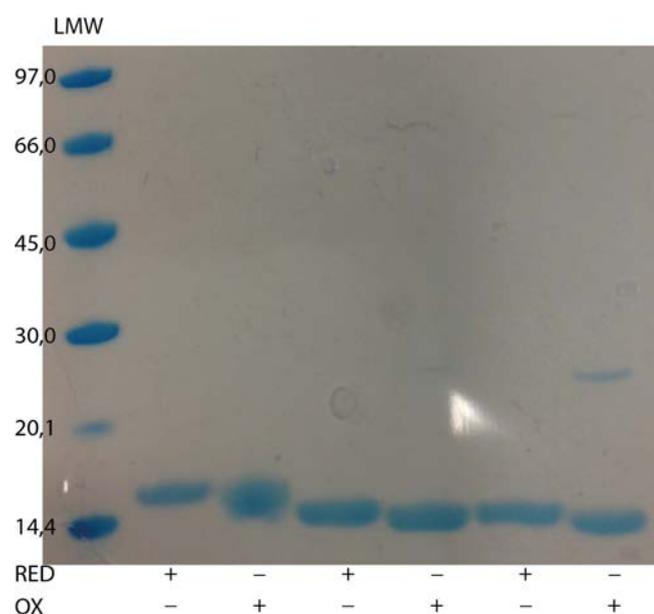
**Table 2.** Mean off-rate ( $k_d$ ) values from two replicates on staphylococcus surface.

Protein	$k_d$ ( $s^{-1}$ )
(ZAb3A12) <sub>2</sub> VE	$2,55 \times 10^{-5}$
ZAbetamatlibSym#25	$1,96 \times 10^{-5}$
ZAbetamatlibSym#57	$1,80 \times 10^{-5}$
ZAbetamatlibSym#73 <sup>a</sup>	$9,54 \times 10^{-6}$
ZAbetamatlibAs#33	$1,49 \times 10^{-5}$
ZAbetamatlibAs#56	$1,53 \times 10^{-5}$
ZAbetamatlibAs#66	$1,01 \times 10^{-5}$
ZAbetamatlibAs50nM#64	$3,37 \times 10^{-5}$
ZAbetamatlibAs50nM#76	$4,74 \times 10^{-5}$

<sup>a</sup>One measurement

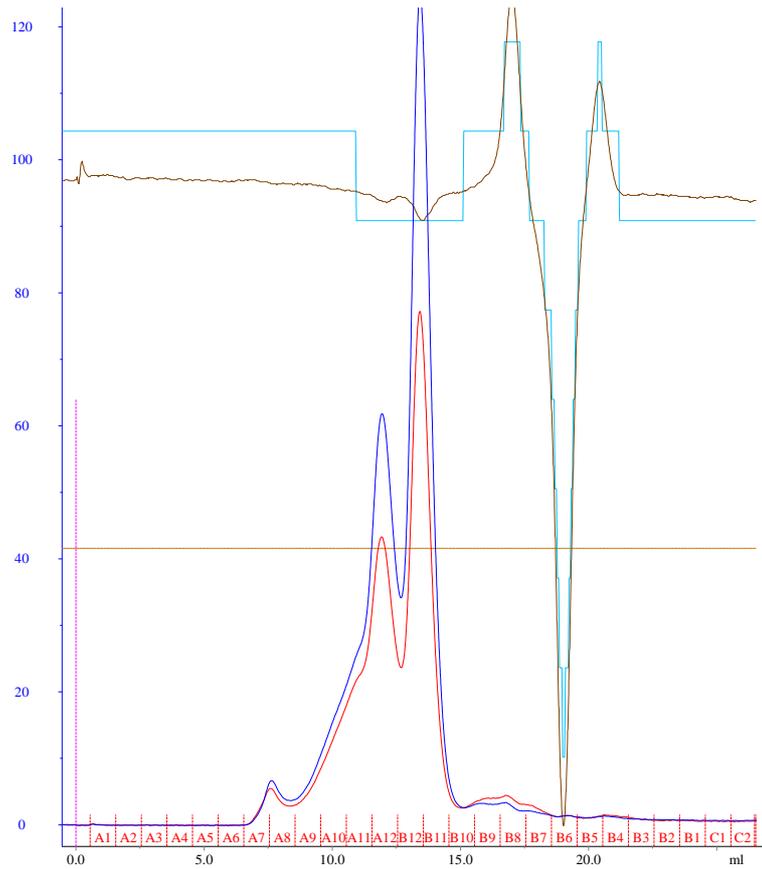
### Evaluation of purity of ZAbetamatlib constructs

The samples that was purified with IMAC and analyzed on a SDS-page gel under oxidative conditions indicated the right length of the purified Affibody molecules and an extra band that was twice the size of the calculated MW of the ZAbetamatlib constructs. The extra band disappeared under redoxidative conditions suggesting dimerization of the Affibody with cysteine-bridge (Figure 7).

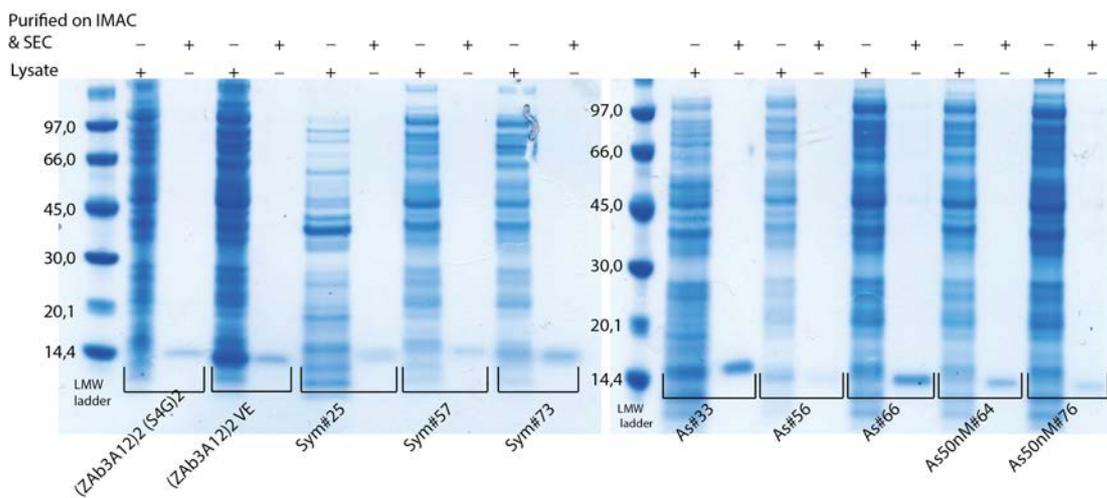


**Figure 7.** SDS gel of three of the IMAC purified ZAbetamatlib constructs during both oxidative- and reducing conditions

During size exclusion chromatography three peaks was observed (Figure 8). The last peak was collected, concentrated and analyzed on SDS-page. After SDS-page analysis the larger band that was seen after IMAC purification could not be detected, suggesting that the SEC purification was successful (Figure 9).



**Figure 8.** Chromatogram from size exclusion purification. Three peaks was observed (red/blue), the first and second from left suggests multimers of the Abetamatlib constructs and the last and highest peak was collected.



**Figure 9** SDS-page image of all purified ZAbetamatlib constructs. Right of each gel is LMW ladder continuing with each construct with lysate and after SEC purification.

MS analysis showed high purity of the proteins but one of the ZAbetamatlib constructs and one control had not the expected size (Table 3).

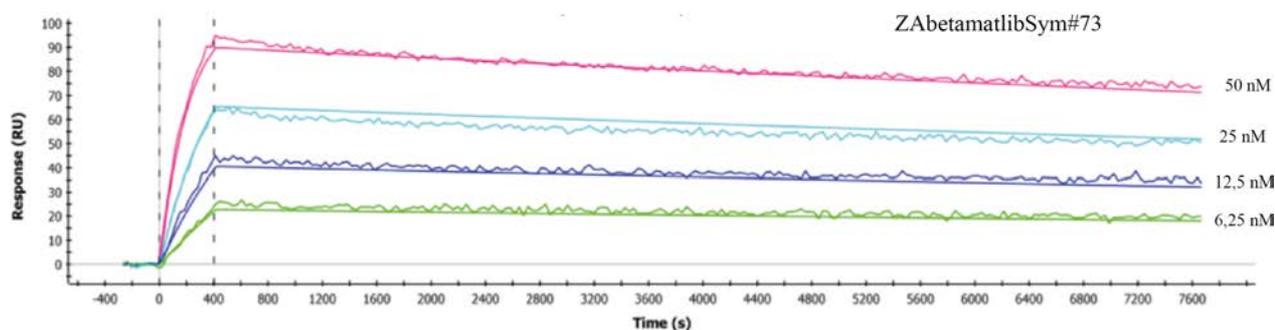
**Table 3.** Production results of ZAbetamatlib proteins

Protein	Length (aa)	Theoretical MW (Da) <sup>a</sup>	Experimental MW (Da) <sup>b</sup>
(ZAb3A12) <sub>2</sub> (S4G) <sub>2</sub>	115	12459,6	12227,9
(ZAb3A12) <sub>2</sub> VE	115	12597,0	12595,2
ZAbetamatlibSym#25	115	12299,4	12299,3
ZAbetamatlibSym#57	115	12420,6	12313,8
ZAbetamatlibSym#73	115	12228,3	12228,5
ZAbetamatlibAs#33	120	12865,0	12862,6
ZAbetamatlibAs#56	115	12312,5	12311,9
ZAbetamatlibAs#66	115	12339,5	12339,5
ZAbetamatlibAs50nM#64	115	12400,5	12400,7
ZAbetamatlibAs50nM#76	115	12291,4	12289,5

<sup>a</sup> Obtained from ExPASy ProtParam tool.  
<sup>b</sup> Obtained from ESI-TOF-MS

### Protein interaction study and kinetics

A protein interaction study was done in BioRads ProteOn to see if the ZAbetamatlib still had affinity for A $\beta$ -peptides and to calculate the kinetic properties of the A $\beta$  binding Affibody molecules. The streptavidin chip was coated with biotinylated A $\beta$  and different concentrations of the Affibody molecules were flown over in two replicates. A non-linear fit was done on each sensogram and the kinetics (Langmuir) was calculated with ProteOn Manager Software. The chromatograms of each construct show again that they had extremely long off-rates and that all of the ZAbetamatlib constructs had over 100 fold better affinity than the original binder,  $3,2 \times 10^{-7}$  (Grönwall *et al.* 2007). One previously published monomeric A $\beta$  binding Affibody molecule was used as a control. In the published data this binder, Z<sub>Ab3</sub>(12-58), had an  $K_D$  of  $6,9 \times 10^{-10}$  in a SPR setting (Lindgren *et al.* 2010) (Table 4, Figure 10, Appendix 2).



**Figure 10.** SPR sensogram from one of the ZAbetamatlib constructs. The time is on the X-axis and the response units on the Y-axis. Between the dotted lines is the injection time and after the second dotted line is the dissociation time, in this case more than 2 hours.

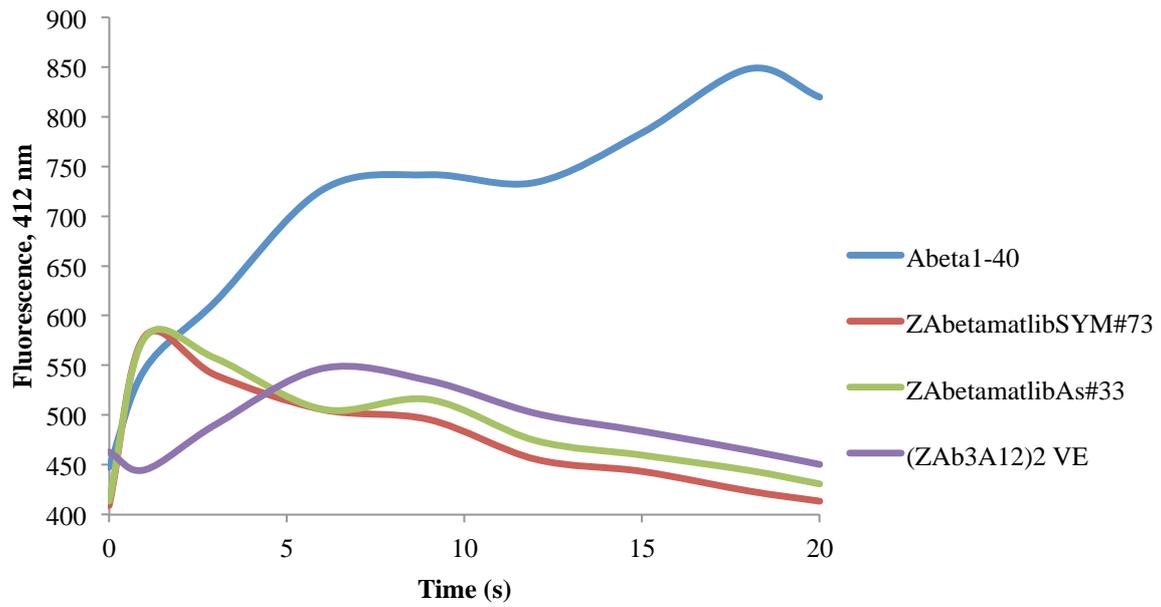
**Table 4.** Mean kinetic data from two replicates for binding of N-terminal Biotinylated Amyloid beta peptides (1-40) to different ZAbetamatlib constructs.

Protein	$k_a$ ( $M^{-1}s^{-1}$ )	$k_d$ ( $s^{-1}$ )	$K_D$ (M)
(ZAb3A12) <sub>2</sub> SG4	$4,85 \times 10^4$	$9,61 \times 10^{-5}$	$1,99 \times 10^{-9}$
(ZAb3A12) <sub>2</sub> VE <sup>a</sup>	$2,27 \times 10^3$	$1,02 \times 10^{-4}$	$4,39 \times 10^{-8}$
ZAbetamatlibSym#25 <sup>a</sup>	$7,16 \times 10^4$	$4,19 \times 10^{-5}$	$5,87 \times 10^{-10}$
ZAbetamatlibSym#57	$1,09 \times 10^5$	$1,48 \times 10^{-4}$	$1,38 \times 10^{-9}$
ZAbetamatlibSym#73 <sup>a</sup>	$9,44 \times 10^4$	$3,16 \times 10^{-5}$	$3,36 \times 10^{-10}$
ZAbetamatlibAs#33	$9,09 \times 10^4$	$8,79 \times 10^{-5}$	$9,68 \times 10^{-10}$
ZAbetamatlibAs#56	$8,84 \times 10^4$	$9,04 \times 10^{-5}$	$1,02 \times 10^{-9}$
ZAbetamatlibAs#66 <sup>a</sup>	$2,77 \times 10^4$	$6,74 \times 10^{-5}$	$2,59 \times 10^{-9}$
ZAbetamatlibAs50nM#64	$1,33 \times 10^5$	$1,53 \times 10^{-4}$	$1,15 \times 10^{-9}$
ZAbetamatlibAs50nM#76 <sup>ab</sup>	$1,62 \times 10^3$	$7,11 \times 10^{-5}$	$6,97 \times 10^{-8}$
Z <sub>Aβ3</sub> (12-58)	$1,56 \times 10^5$	$2,14 \times 10^{-4}$	$1,38 \times 10^{-9}$

<sup>a</sup> 7600 seconds dissociation time in SPR experiments.  
<sup>b</sup> Data obtained from a 100 RU immobilization surface.

### Aggregation assay

This analysis was in the trial-phase and no optimizations were done. Aβ<sub>1-42</sub> was mixed with a few of the binders together with ThT and 1 x PBS. Fluorescence was noted every 15 minutes for 20 hours. The results indicate however that the Amyloid beta binding Affibody molecules were inhibiting the formation of Aβ-plaques (Figure 11).



**Figure 11.** ThT assay with two of the ZAbetamatlib constructs and one of the control-binders. Blue line represents Amyloid beta peptides without Affibody molecules present. Red line represents Amyloid beta peptides together with ZAbetamatlibSym#73. Green line represents Amyloid beta peptides together with ZAbetamatlibAs#33. Purple line represents Amyloid beta peptides together with one of the control-binders, (ZAb3A12)2 VE.

## Discussion

As the presence of high levels of the 42 amino acid long variant of the A $\beta$ -peptide is one of the theories around Alzheimer's disease the reduction of this peptide could be important as a therapy for these patients. Many therapies have been tried out but now a day there is not much hope for the Alzheimer's patients. The most promising drug trial in several years was Bapineuzumab, a humanized antibody. However, this drug did not have the effect that the provider hoped for. So, it is important to find new strategies to suppress the toxic effect that the A $\beta$ -plaques provide. One of the strategies that had been tried is depletion of the peptide with the peripheral sink mechanism and altering the equilibrium of the peptide. Studies have shown that this has an effect of the levels of A $\beta$  in the central nervous system. Instead of using antibodies that can trigger an immune reaction you can use another affinity protein, the Affibody molecule, which in some cases have several advantages; as it is smaller and folds quickly and it can be made with chemical synthesis and they may not trigger the immune system in a way that antibodies does. The small size affinity protein may pass the blood-brain-barrier in the same way and target protein within the CNS. So, in an A $\beta$  objective this is perfect. When the A $\beta$  Affibody molecule is in the blood it can take free Amyloid beta peptides and clear them, but at the same time they might penetrate the CNS and hinder the plaque formation that gives a double effect.

## Flow-cytometric studies

As expected all the A $\beta$  binding Affibody molecules that was given to me had affinity towards the A $\beta$ -peptide. We can see in figure 3 that all of them showed a higher signal than the original binders in its dimeric form that was used as a control. This suggests that the protein in its free form also had higher affinity, which was the goal for engineering the different variants.

The off-rate study showed that the different affinity proteins hold on to the target differently and the off-rate of the Amyloid beta is different. The reason for gating the binding population in the dotplots can be observed in figure 4. As the labeled A $\beta$  was released is got sticky and unspecific binding could be observed. These dots that represents an event thereby alter the median value that was later used for the calculations. As the gating was done manually in you could say that the results are altered. However, the gating was done without any regards to the previous time point and in an objective manner. In order to reduce the non-specific binding more washes could be applied to the protocol. The study was done in two replicates and showed good reproducibility, which suggest that the off-rates that were gained are relevant. However, the slow off-rates and the few time points made the non-linear regression fit to the plotted values hard. Some of the regressions fitted well and some differed a bit (Appendix 1). Maybe by adding more time points a better fit can be done but this require that several people preform this experiment and more than one centrifuge are in use since the different steps in the time points collides.

## Production of Amyloid beta binding Affibody molecules

The first step of the production was the polymerase chain reaction, and to amplify the gene that was in the pScZ1 vector. Since the gene is a dimer there were unspecific binding of the primers. This lead to that two populations of DNA was amplified, one with a monomeric variant and one with the dimeric variant. These two populations travels in different speed on a gel since they differ in size. In order to differentiate these two populations a gelextraction was done. Here, the band with the correct length was cut out of the gel with a scalpel and purified with a gel purification kit. When the different constructs were purified they were ligated into the pET26b+

vector, one negative sample was used with only vector and no insert. All the constructs were ligated in a manner that they will be produced in the cytoplasm. This is usually the protocol for producing the Affibody molecule since it has shown to be a very stable molecule. However, in the last part of the project we decided to see if the yield for production would increase if they were produced on the cell surface. So, new primers were designed for two of the constructs. These primers were designed in a manner that the DNA fragment could be ligated in a different place in the vector, behind the pectate lyase B (*pelB*) sequence. This leader sequence will direct the Affibody molecule to the periplasm and the Affibody molecule can be cleaved off (Lei *et al.* 1987). All of the constructs together with the constructs that were fused to *pelB* were transformed to *E.coli* RR1 cells and spread on a TBAB plate containing kanamycin. The number of colonies on the negative plate (from the ligation step) showed that there was a low background, where the vector had ligated to it self. Several colonies from the different constructs were picked and a PCR screen was performed, at the same time the colony was re-stroked on a new plate. This step was very time consuming because the protocol had to be optimized. Before optimization there was a lot of primer dimers and in some cases there was no amplification at all. After optimization the number of primer dimer was reduced but they were still present. When I was able to get a PCR product that was in the right size I did a ethanol precipitation to purify the DNA. This DNA was then sequenced and the sequences were analyzed with Geneious software. Also this step were time consuming since many of the sequences were unreadable. The sequences that were readable and had the right sequence were noted and the colony that corresponded with the right sequence was inoculated. A miniprep was done on these in order to lyse the cells and to retain the plasmid. This was then transformed into protein producing *E.coli* BL21 cells. One colony from each construct was picked and inoculated and the protein production began by inducing with IPTG.

As mentioned earlier, these Affibody molecules contain cysteine and in order for the molecule to retain its structure with the cysteine-bridge a native purification was optimal. By lysing the cells with sonification, as our first chose was, the cysteine-bridge will remain intact due to the non-reducing buffer. This type of purification worked for some of the constructs and for some it did not work, and a very small amount of protein could be purified from IMAC. As the proteins were going to be purified further with size exclusion and thereby diluted further, another purification method was used in order to increase the yield. A much higher amount of protein was gained when purifying with denaturizing conditions, where you lyse the cells chemically. This did not seem to do any difference in the later size exclusion chromatography. In some cases the lysing under denaturizing conditions did not work either. As a last resort these cells were lysed with French-press. This increased the yield a lot and a lot of protein was retrieved with this method. I think that when these proteins are going to be produced in the future the french-press is a good alternative since the yield was increased and it is still under native conditions.

The SDS-page showed that there was some impurity with extra bands with twice the size of the Affibody molecule. The theory to these bands is that the cysteine-bridge within the Affibody molecule is broken and is retained between two of the Affibody molecules. These multimers must be removed and this was done with size exclusion chromatography. This was also a step that was very time consuming; the column that was use could not be run under a high pressure so the flow rate had to be reduced. The chromatogram (Figure 8) showed three peaks, and the reason for these three is the multimerization of the Affibody molecule in the same manner as described before. The last peak and the Affibody molecules with the retained structure were collected. The sample size for the column was 0,5 ml so each construct had to be ran in rounds, all fractions that contained the third peak was pooled and later concentrated since the volume was to big. The column that was used made the samples impure with some extra bands that were

slightly bigger than the Affibody molecule suggesting some additional molecules that are attaching to the Affibody molecule, maybe to one of the cysteine. The extra size was also confirmed with mass spectrometry. This problem was however solved by repacking of the column with the same matrix that was used before. The matrix was however cleaned with ethanol and iso-propanol. So, all the samples that showed this extra band was produced and IMAC purified again. A note that can be done is the size of the column, it may be a bit too short since there are unresolved peaks and they overlap. So for future purification a larger column is preferred.

As these proteins seem to be prone to multimerize with the cysteine the stability is questionable. If the cysteine-bridge is stable it may take a long time before they multimerize again but if it is weak it may already have happened since it is moving towards equilibrium where both multimers and single Affibody molecules are present.

### **Verification of size**

Between each step of the purification a SDS-page was run which showed the approximate size and purity of each Affibody molecule. The mass spectrometry showed later that all of the constructs, except two, had the same size as the calculated one. The reason for the smaller size that could be seen is unclear. BL-21 cells do however truncate proteins some times. I tried to calculate a new molecular weight by removing amino acids in the end to see if this was the case. The new calculated did not correlate with the actual size and the reason for this reduction in size is still unknown. It was not enough time to produce these again so we continued with these two anyway.

### **Protein interaction studies**

The reason for doing this study on BioRads ProteOn compared to the Biacore SPR that also was available was that you could measure on six different surfaces in one chip with ProteOn. Five different surfaces were immobilized with different amounts of biotinylated A $\beta$  and one of the surfaces was not coated and was used as a control and the signal was stabilized with this. All of the purified protein was diluted and five different concentrations of each construct were flown over the A $\beta$ -peptides. This was done in order to do proper calculations of the kinetics. Each construct was run twice and a mean value was calculated from this. This gave a very low standard deviation (data not shown) so they are representable. All constructs were not purified at the same time so the chip had to be stored in the fridge until the last ones were done. This may have affected the results and some A $\beta$ -peptide may have been released from the streptavidin chip. The constructs that were run before storing were analyzed on a surface that was immobilized with 50RU A $\beta$ . The last ones were analyzed on the surface that was immobilized with 100RU due to this.

As a control a previously published A $\beta$  binding Affibody was used. The affinity of this binder in my studies,  $1,38 \times 10^{-9}$  M, compared to the published affinity,  $6,9 \times 10^{-10}$  M, suggest that the affinity measurements are representable and good. Previous SPR studies on this binder were done on Biacore so that may be the reason for the small difference. Also, when inspecting the chromatogram you see that the linear fit is not optimal (Appendix 2). All binders had very good affinity towards A $\beta$ -peptides. Also, the off-rate is very long which is good in this case. The affinity for these binders has over a 100-fold improvement compared to the original binder Z<sub>Ab3</sub> that had a  $K_D$  value of  $3,20 \times 10^{-7}$  M.

### **Aggregation studies**

As mentioned before the inhibition of plaque formation could be one important aspect in order to treat AD. To investigate this *in vitro* a Thioflavin T assay was performed. ThT binds to aggregates of A $\beta$ -peptides, plaques. When binding, it emits a light that can be monitored. This was the last step of my work and at this stage time was a limiting factor. The assay has not been optimized and this result is more of an indication. In figure 11 we can see that the fluorescence is increased over time when only the A $\beta$ -peptide is present, suggesting that there is some plaque formation. We can also observe that the fluorescence does not increase over time when the A $\beta$  binding Affibody molecule is present, indicating that it inhibits the plaque formation. The reason for the increase of fluorescence at the beginning of the measurements is not known but it may be due to the plate reader and I am sure that this assay can be optimized in a manner that it will disappear.

### **Comparing off-rate on Staphylococcus surface and in free form**

The off-rates differ a bit depending on if the protein is in its free form or on the staphylococcus surface. This may be due to several reasons; one may be that they actually behave differently, another reason can be that on the staphylococcus surface the binder is fixed and this may alter the binding properties, also the ABD domain that is fused to the binder may interfere. Further is that in the off-rate studies on Staphylococcus surface the A $\beta$ -peptide was in free form compared to the SPR experiment where it was immobilized. Also, since the off-rates are very long a calculation from the non-linear fit is hard and the adaption of the curve to measure points is in some cases poor (Appendix 1).

## Conclusions

In this project we can conclude that it is possible to produce and purify a head-to-tail engineered A $\beta$  binding Affibody molecule even though it contains cysteine. Some of the given protocols had to be optimized to fit this project and I am sure that they can be optimized even further. One of the desires may be to increase the yield when purifying the protein but french-press seemed to be one good alternative. Also, trying to produce these molecules in the periplasm could increase the yield. A larger column is also preferable when purifying these proteins in order to reduce the unresolved peaks.

We can also conclude that the affinity towards the A $\beta$ -peptide is high and that the off-rates are very long. In the best binder the affinity was measured up to  $9,68 \times 10^{-10}$  M and the best off-rate was measured to  $9,68 \times 10^{-10} \text{ s}^{-1}$ . This together with the indication from the ThT assay that the Affibody molecule inhibits the progression of plaque formation makes these binders suitable in a therapeutically setting.

In the nearest future the secondary structure of these binders is going to be investigated with Circular Dichroism. This will also show how fast these proteins refold and a  $T_m$  can be obtained. Later, some of these are going to be tried out in mouse models where both the therapeutically function will be tested but it will also be used as a imaging tool.

The aim of engineering the two new maturation libraries of the Amyloid beta binding Affibody molecule was to improve the affinity towards the Amyloid beta peptides and goal has been achieved. My studies show that the affinity has been improved over 100 fold compared to the original Z<sub>Ab3</sub>.

## Acknowledgement

First of all I would like to thank my supervisor Hanna Lindberg. You have made this a very fun and interesting project and I am very grateful for all the answers you have given me and for all the help. This project was very well thought of and your ability to organize inspires me. I would really like to thank my co-supervisor John Löfblom for all the help he has given me in the lab and also for helping me with the analysis of all results. I would like to thank Joel Lindgren for the Amyloid beta binding Affibody molecule that was used as a control in the ProteOn experiment. I would also like to thank “the protein factory” for helping me with BCA and MS. Also, I would like to thank my opponent, Ken Andersson. Lastly, I would really like to thank Professor Stefan Ståhl for accepting me to this project.

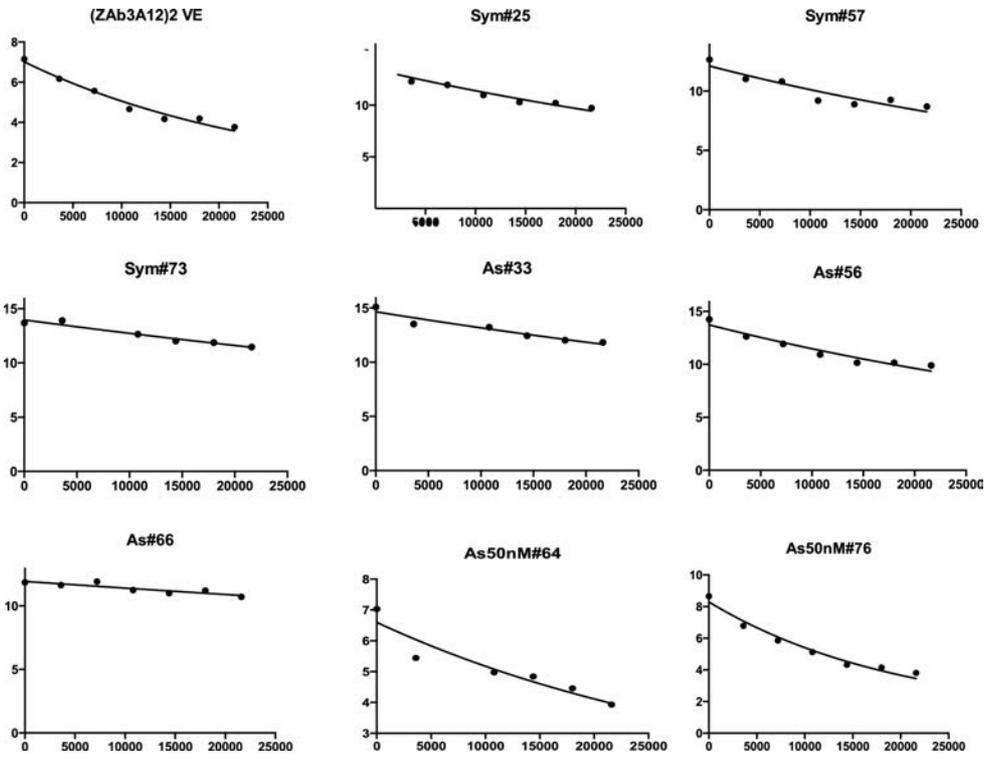
## Reference:

- Alzheimer's Association. 2009. 2009 Alzheimer's disease facts and figures. *Alzheimer's and Dementia* **5**: 234-270
- Alzheimer's Association. 2012. WWW-document: <http://www.alz.org>. Collected: 2012-06-14
- Augustin J and Gotz F. 1990. Transformation of *Staphylococcus epidermidis* and other staphylococcal species with plasmid DNA by electroporation. *FEMS Microbiology Letter* **54**: 203-7.
- Behr D, Hesse L, Masters CL and Multhaup G. 1996. Regulation of Amyloid Protein Precursor (APP) Binding to Collagen and Mapping of the Binding Sites on APP and Collagen Type I. *Journal of biological chemistry* **273**: 1613-1620
- Danysz W and Parsons CG. 2003. The NMDA receptor antagonist memantine as a symptomatological and neuroprotective treatment for Alzheimer's disease: preclinical evidence. *International Journal of Geriatric Psychiatry* **18**: S23-S32
- Delacourte A and Defossez A. 1986. Alzheimer's Disease: Tau Proteins, the promoting Factors of Microtubule Assembly, are Major Components of Paired Helical Filaments. *Journal of the Neurological Sciences* **76**:173-186.
- DeMattos RB, Bales KR, Cummins DJ, Dodart J-C, Paul SM, and Holtzman DM. 2001. Peripheral anti-A $\beta$  antibody alters CNS and plasma A $\beta$  clearance and decreases brain A $\beta$  burden in a mouse model of Alzheimer's disease. *PNAS* **98**: 8850-8855.
- Geling A, Steiner H, Willem M, Bally-Cuif L and Haass C. 2002. A  $\gamma$ -secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO reports* **3**, 7, 688-694.
- Gilman S, Koller M, Black RS, Jenkins L, Griffith SG, Fox NC, Eisner L, Kirby L, Rovira MB, Forette F and Orgogozo JM. 2005. Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. *Neurology* **64**: 1553-62.
- Hardy JA and Higgins GA. 1992. Alzheimer's disease: The amyloid cascade hypothesis. *Science* **256**: 184-185.
- Hoyer W, Grönwall C, Jonsson A, Ståhl S, and Härd T. 2008. Stabilization of a  $\beta$ -hairpin in monomeric Alzheimer's amyloid- $\beta$  peptide inhibits amyloid formation. *PNAS* **105**: 5099-5104.
- Johnson & Johnson Corp. 2012. WWW-document <http://www.jnj.com/connect/news/all/johnson-and-johnson-announces-discontinuation-of-phase-3-development-of-bapineuzumab-intravenous-iv-in-mild-to-moderate-alzheimers-disease>. Retrieved: 2012-10-29.
- Jonsson A, Grönwall C, Lindström S, Gunneriusson E, Ståhl S, Herne N. 2007. Selection and characterization of Affibody ligands binding to Alzheimer amyloid  $\beta$  peptides. *Journal of Biotechnology* **121**: 162-183.
- Kaden D, Munter LM, Joshi M, Treiber C, Weise C, Bethge T, Voigt P, Schaefer M, Beyermann M, Reif B and Multhaup G. 2008. Homophilic interactions of the amyloid precursor protein (APP) ectodomain are regulated by the loop region and affect beta-secretase cleavage of APP. *Journal of Biological Chemistry* **283**: 7271-7279
- Korotzer AR, Pike CJ and Cotman CW. 1993.  $\beta$ -amyloid peptides induce degeneration of cultured rat microglia. *Brain Research* **624**: 121-125.
- Kronqvist N, Löfblom J, Jonsson A, Wernérus H and Ståhl S. 2008. A novel affinity protein selection system based on staphylococcal cell surface display and flow cytometry. *Protein Engineering, Design and Selection* **21**: 247-255
- Lei SP, Lin HC, Wang SS, Callaway J, and Wilcox G. 1987. Characterization of the *Erwinia carotovora pelB* gene and its product pectate lyase. *J Bacteriol.* **169**: 4379-4383.

- Li J, Lundberg E, Vernet E, Larsson B, Höidén-Guthenberg I, Gräslund T. 2012. Selection of affibody molecules to the ligand-binding site of the insulin-like growth factor-1 receptor. *Biotechnology and Applied Biochemistry* **55**: 99–109.
- Lindberg H, Johansson A, Härd T, Ståhl S and Löfblom J. 2012. Staphylococcal display for combinatorial protein engineering of a head-to-tail affibody dimer binding the Alzheimer amyloid- $\beta$  peptide. *Biotechnology Journal* DOI: 10.1002/biot.201200228
- Lindgren J, Wahlström A, Danielsson J, Markova N, Ekblad C, Gräslund A, Abrahmsén L, Karlström AE and Wärmländer SKTS. 2010. N-terminal engineering of amyloid- $\beta$ -binding Affibody molecules yields improved chemical synthesis and higher binding affinity. *Protein Science* **19**: 2319–2329
- Lim KH, Collver HH, Le Y T.H, Nagchowdhuri P and Kenney JM. 2007. Characterizations of distinct amyloidogenic conformations of the A $\beta$  (1–40) and (1–42) peptides. *Biochemical and Biophysical Research Communications* **353**: 443-449.
- Luheshi LM, Hoyer W, de Barros TP, van Dijk Härd I, Brorsson A-C, Bertil Macao, Persson C, Crowther DC, Lomas DA, Ståhl S, Dobson CM, Härd T. 2010. Sequestration of the A $\beta$  Peptide Prevents Toxicity and Promotes Degradation In Vivo. *PLoS Biol* 8(3): e1000334. doi:10.1371/journal.pbio.1000334
- Lyakhov I, Zielinski R, Kuban M, Kramer-Marek, G, Fisher R, Chertov O, Bindu L and Capala J. 2010 HER2- and EGFR-specific affiproboscopes: novel recombinant optical probes for cell imaging. *Chembiochem* **11**: 345–350.
- Myers JK and Oas TG. 2001. Preorganized secondary structure as an important determinant of fast protein folding. *Nature Structural Biology* **8**: 552-558.
- Pike CJ, Burdick D, Walencewicz AJ, Glabe CG and Cotman CW. 1993. Neurodegeneration Induced by  $\beta$ -amyloid Peptides in vitro: The Role of Peptide Assembly State. *The Journal of Neuroscience* **13**: 1676-1687.
- Priller C, Bauer T, Mitteregger G, Krebs B, Kretschmar HA and Herms J. 2006. Synapse formation and function is modulated by the amyloid precursor protein. *Journal of Neuroscience* **26**: 7212–21.
- Rüther U. 1982. pUR 250 allows rapid chemical sequencing of both DNA strands of its inserts. *Nucleic Acids Res.***10**: 5765-5772.
- Salloway S, Sperling R, Gilman S, Fox NC, Blennow K, Raskind M, Sabbagh M, Honig LS, Doody R, van Dyck CH, Mulnard R, Barakos J, Gregg KM, Liu E, Lieberburg I, Schenk D, Black R and Grundman M. 2009. A phase 2 multiple ascending dose trial of bapineuzumab in mild to moderate Alzheimer disease. *Neurology* **73**: 2061-70
- Samuelson P, Hansson M, Ahlborg N, Andréoni C, Götz F, Bächli T, Nguyen TN, Binz H, Uhlén M and Ståhl S. 1995. Cell surface display of recombinant proteins on *Staphylococcus carnosus*. *Journal of bacteriology*: 1470-1476
- Sgourakis NG, Yan Y, McCallum S, Wang C, and Garcia AE. 2007. The Alzheimer's peptides A $\beta$ 40 and 42 adopt distinct conformations in water: A combined MD / NMR study. *Journal of Molecular Biology* **368**: 1448–1457.
- Smith P G. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**: 1315-1317.
- Studier FW. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol.* **89**: 113-30.
- U.S National institute of health. 2012. Clinical trials, WWW-document: [clinicaltrials.org](http://clinicaltrials.org) Retrieved: 2012-10-29
- World Health Organisation. 2004. Alzheimer's disease. WWW-document: [http://www.searo.who.int/en/section1174/section1199/section1567\\_6740.htm](http://www.searo.who.int/en/section1174/section1199/section1567_6740.htm). Retrieved: 2012-05-22

# Appendix 1.

Median values from flow-cytometry plotted against time and a non-linear regression fit for all constructs.



## Appendix 2.

Sensograms from ProteOn.

